A protein from the salivary glands of the pea aphid, *Acyrthosiphon pisum*, is essential in feeding on a host plant

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In feeding, aphids inject saliva into plant tissues, gaining access to phloem sap and eliciting (and sometimes overcoming) plant responses. We are examining the involvement, in this aphid–plant interaction, of individual aphid proteins and enzymes, as identified in a salivary gland cDNA library. Here, we focus on a salivary protein we have arbitrarily designated Protein C002. We have shown, by using RNAi-based transcript knockdown, that this protein is important in the survival of the pea aphid (*Acyrthosiphon pisum*) on fava bean, a host plant. Here, we further characterize the protein, its transcript, and its gene, and we study the feeding process of knockdown aphids. The encoded protein fails to match any protein outside of the family Aphididae. By using *in situ* hybridization and immunohistochemistry, the transcript and the protein were localized to a subset of secretory cells in principal salivary glands. Protein C002, whose sequence contains an N-terminal secretion signal, is injected into the host plant during aphid feeding. By using the electrical penetration graph method on *c002*-knockdown aphids, we find that the knockdown affects several aspects of foraging and feeding, with the result that the *c002*-knockdown aphids spend very little time in contact with phloem sap in sieve elements. Thus, we infer that Protein C002 is crucial in the feeding of the pea aphid on fava bean.

aphid–plant interaction | saliva | RNAi | electrical penetration graph | immunohistochemistry

The ability, or inability, of an aphid to feed on a plant results from a multifaceted interplay between the feeding systems of the insect and the defense systems of the plant (for recent reviews, from several perspectives, of aphid–plant interactions, see refs. 1–5). When, in an ongoing coevolution, the aphid has (temporarily) established an advantage, it can probe to and enter its feeding site, the sieve element containing phloem sap, the aphid's source of nutrients; can overcome the plant's defense, possibly during probing and certainly during its extended feeding on the phloem sap; and will eventually withdraw from the plant, having, at least in some cases, not caused great damage. This sequence of events establishes the host range of the aphid. From the standpoint of this article, it is important to note that salivation continues throughout the process, including the extended feeding on phloem sap (3).

Despite decades of investigation, the steps or phases in aphid feeding remain poorly understood at a molecular level. Recently, however, Will *et al.* (6) have identified a system in which they have gained considerable insight at the molecular level. In particular, they demonstrated that calcium-binding proteins of aphid saliva may undermine a calcium-requiring mechanism of plant defense. Such aphid proteins, in binding Ca^{2+} in phloem sap, would cause a conversion of the proteinaceous "forisomes" of sieve elements to a contracted (nonblocking) state, thus preventing the forisomes from occluding the sieve tubes, which the forisomes would otherwise do to prevent a continued leakage of phloem sap through the puncture wound from the aphid's stylets. At this point, the individual proteins involved in this undermining of this plant defense are unidentified, but the work of Will *et al.* (6) illustrates well that the saliva of aphids likely holds the secret to many aspects of aphid–plant interaction.

Aphid saliva has therefore received a good deal of attention. Until recently, most studies have been at an enzymatic level. This large body of work has been comprehensively reviewed by Miles (7). Summarized briefly, two broad types of enzymes can be expected in the saliva of any aphid, namely oxidoreductases and hydrolases. Among the first type, polyphenol oxidase and "peroxidase" are of special note because of their occurrence in many aphid species and their centrality in Miles' "redox hypothesis" of detoxification of defensive phytochemicals. The most commonly reported hydrolases are carbohydrases, such as cellulase, sucrase, and pectinases. The difficulties faced in such work are, however, considerable, given the minute amounts of saliva produced, and the result is that little is known with any degree of certainty about the biochemical composition of any given aphid's saliva.

Accordingly, we have opted for a molecular genetics approach; that is, we aim to access, and then study, individual proteins of saliva through salivary gland cDNA libraries. From an initial examination of ≈4,700 ESTs from such a library, we have selected one contig for detailed investigation. It was arbitrarily designated transcript *c002* and was selected on the basis of its abundance (it was the seventh most populous EST contig) and the presence of a full open-reading-frame, encoding a small protein, of 219 residues, which contains a predicted signal peptide for secretion at its N terminus. We have reported that RNAi-based *c002* transcript knockdown dramatically reduces the life span of pea aphids on fava bean leaves (8).

Here, we report on the transcript *c002*, its gene, and the encoded protein, including localization of the transcript and protein in a subset of secretory cells in the principal salivary glands and the transfer of Protein C002 to the host plant during aphid feeding. Using the electrical penetration graph (EPG) method (3), we have studied the feeding behavior of pea aphids in which transcript *c002* has been knocked down. From these

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AAAAGTTTGTTCAAACAAATATCTCGTCGTGTATCCAGTGCGATAGCGATAATTTACAAC    60
ATGGGAAGTTACAAATTATACGTAGCCGTCATGGCAATAGCCATAGCTGTAGTACAGGAA   120
 M  G  S  Y  K  L  Y  V  A  V  M  A  I  A  I  A  V  V  Q  E
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 V  R  C ↑D  W  S  A  A  E  P  Y  D  E  Q  E  E  A  S  V  E
TTACCGATGGAGCACCGTCAGTGCGATGAATACAAATCGAAGATCTGGGACAAAGCATTT   240
 L  P  M  E  H  R  Q  C  D  E  Y  K  S  K  I  W  D  K  A  F
AGCAACCAGGAGGCTATGCAGCTGATGGAACTAACGTTTAATACAGGTAAGGAATTAGGC   300
 S  N  Q  E  A  M  Q  L  M  E  L  T  F  N  T  G  K  E  L  G
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 S  H  E  V  C  S  D  T  T  R  A  I  F  N  F  V  D  V  M  A
ACCAACCAGAACGCCCATTACTCGCTGGGTATGATGAACAAGATGTTGGCGTTCATCATA   420
 T  N  Q  N  A  H  Y  S  L  G  M  M  N  K  M  L  A  F  I  I
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 R  E  V  D  T  T  S  N  K  F  K  E  T  K  E  V  F  E  R  I
GCGAAAACTCCAGAGATCCGAGACTATATCAAGCACACGACCGCCCGGACCGTCGACTTG   540
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CTCAAAGAGCCCGTGATTAGAGGCCGACTGTTCAAAGTGGTGAAAGCCTTCGAGGGTCTG   600
 L  K  E  P  V  I  R  G  R  L  F  K  V  V  K  A  F  E  G  L
ATAAAACCGTCCGAAAACGAGGAATTGGTCAAGCAGAGGCTTAAGAGGATAACCAATGCT   660
 I  K  P  S  E  N  E  E  L  V  K  Q  R  L  K  R  I  T  N  A
CCCGCCAAGATGGCTATGGGAGCCATAAATAAGTTTGGAAGTTTCCTTCGACGTTTTTA    720
 P  A  K  M  A  M  G  A  I  N  K  F  G  S  F  L  R  R  F  *
ATAAGCGCGTCCATACAGACTAGTGATATATTATATATATATACTTATAA             770
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Fig. 1. Nucleotide sequence of transcript *c002* and the inferred amino acid sequence of Protein C002. The nucleotide sequence is a unigene sequence assembled from *c002* ESTs from NCBI. It agrees with the sequence obtained from the *c002* gene (see Fig. S1). The arrow indicates the signal peptide cleavage site as predicted by SignalP.

studies we conclude that Protein C002, a secreted component of salivary glands, is essential for feeding on fava bean by the pea aphid.

Results

Sequences of Transcript *c002* and Protein C002. In Fig. 1 we present the unigene nucleotide sequence of transcript *c002* and its predicted encoded amino acid sequence. The predicted protein contains 219 aa residues. The N-terminal sequence of the protein is predicted by SignalP to be a signal peptide for an extracellular protein (www.cbs.dtu.dk/services/SignalP/), with cleavage predicted between residues 23 and 24. The mass of the predicted mature protein is 21.8 kDa. There are no potential O-glycosylation sites (www.cbs.dtu.dk/services/NetOGlyc/) or N-glycosylation sites (www.cbs.dtu.dk/services/NetNGlyc/). Programs for prediction of secondary structure predict a high helix content interrupted by loops or turns. For instance, the PROF program of Rost *et al.* (9) predicts 62% helix in the mature protein and on this basis tentatively classifies the Protein C002 as "all-α."

c002 is an abundant EST in our salivary gland cDNA library. In ≈4500 ESTs from the library, there are 17 occurrences of the *c002* EST, a 14-fold higher frequency than among head and whole-body ESTs (even though these include a normalized whole-body library), thus indicating an enrichment of the cDNA in the salivary gland library.

Blasting *c002* against the nonredundant database and the EST database at National Center for Biotechnology Information (NCBI) reveals no strong matches to any protein of known function or indeed to any predicted protein at all outside of the family Aphididae. Homologs are found among aphids, including the brown citrus aphid, *Toxoptera citricida*, the cotton aphid, *Aphis gossypii*, the green peach aphid, *Myzus persicae*, and the greenbug, *Schizaphis graminum* (10). An analysis of variation of the C002 sequence in several aphid species will be presented elsewhere (J.M., unpublished results).

Transcript Size and Gene Copy Number. Northern blot analysis of total pea aphid RNA using a full-length *c002* probe revealed a single band of 1,100 bases (Fig. 2 *Left*). In Southern analysis, we observed single bands in digests by several enzymes, suggesting a single gene (locus) encoding Protein C002 (Fig. 2 *Right*). Assembly of the *c002* gene from the genomic reads available at

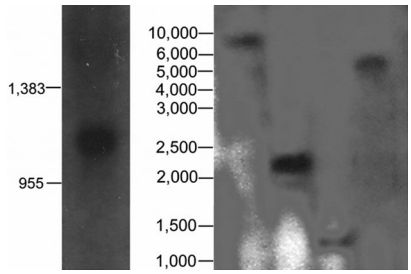

Fig. 2. Northern and Southern blot analyses of the *c002* transcript and gene. (*Left*) Northern blot for the *c002* transcript. (*Right*) Southern blot of pea aphid genome DNA digested by: XbaI (first lane), NcoI (second lane), EcoRV (third lane), and EcoRI (fourth lane).

the Baylor College of Medicine Human Genome Sequencing Center (www.hgsc.bcm.tmc.edu/projects/aphid/) indicates a single gene, containing a single intron [see supporting information (SI) Fig. S1].

Localization of Transcript *c002* and Protein C002 Within Salivary Glands. Most of the volume of aphid salivary glands is comprised of large secretory cells, 4 in each accessory gland and 21 in each lobe of the bilobed principal gland (11, 12). *In situ* hybridization with digoxygenin-labeled *c002* DNA revealed the presence of transcript *c002* only in the principal salivary glands and in only a few of the secretory cells in each lobe (Fig. 3 *A* and *B*). The unstained (differential interference contrast) image shows the overall morphology expected of an accessory salivary gland and its associated (bilobed) principal salivary gland (11, 12). (These authors' diagrams of the histology of aphid salivary glands can be seen online, on pages 51 and 53 in ref. 10). Propidium iodide staining revealed the large nuclei expected within the secretory cells, and the *c002* transcript was largely confined to a handful of such cells in each lobe of the principal gland.

Immunohistochemical localization of Protein C002, using rabbit polyclonal antibodies raised against the recombinant protein, revealed staining in a subset of several cells in the principal salivary gland (Fig. 3*C*).

Detection of Protein C002 in Plants Fed upon by Aphids. Five hundred aphids were placed on fresh fava bean plants for 24 h and then removed. Leaf tissue was then extracted and Western blot analysis was performed using polyclonal anti-C002 rabbit antibodies. As shown in Fig. 4 (lane 3), a band was detected in plants fed upon by aphids that matches the position of recombinant Protein C002 (Fig. 4, lane 1). On the other hand, a Protein C002 band was not detected in plants that had not been exposed to

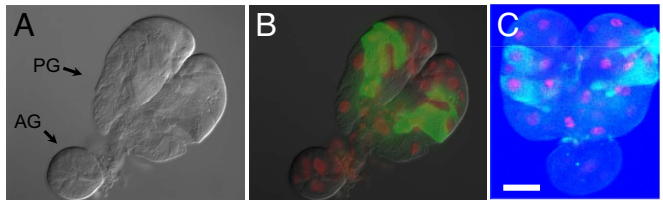

Fig. 3. *In situ* hybridization and immunohistochemical localization of Protein C002 in dissected pea aphid salivary glands. (*A*) Differential interference contrast image. PG, principal gland; AG, accessory gland. (*B*) Differential interference contrast image overlaid with *in situ* hybridization for transcript *c002*. Green is the positive signal for antisense *c002*; nuclei are red. (*C*) Immunohistochemistry with anti-Protein C002 staining. Nuclei are red; green is the positive signal for anti-C002 antibody. (Scale bar: 50 μm.) Negative controls (sense probe for *in situ* hybridization and preimmune serum for the immunohistochemistry) did not show any positive staining.

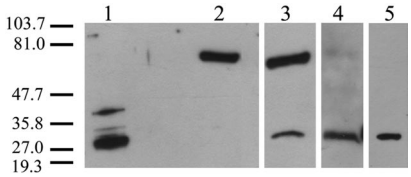

Fig. 4. Detection of Protein C002 in fava bean extract after aphid feeding. Rabbit anti-C002 polyclonal antibodies were used to develop Western blots. Samples for electrophoresis were as follows: recombinant Protein C002 (lane 1); extract of fava bean tissue, without aphid feeding (lane 2); extract of fava bean tissue, after pea aphid feeding (lane 3); extract from five pea aphid heads (lane 4); extract from five dissected pea aphid salivary glands (lane 5). The experiment was carried out four times with similar results. Further negative controls, beyond lane 2), were as follows. Omission of primary antibody resulted in a blank lane (no bands). Use of preimmune serum, rather than the primary antibody, resulted in a single band in the plant samples of ≈75 kDa, regardless of aphid feeding.

aphids (lane 2). These results indicate that Protein C002 is transferred from aphid to plant during feeding. Protein C002 was also detected in extracts from pea aphid heads and salivary glands (lanes 4 and 5, respectively). No bands were detected in any of these samples in a Western blot with secondary antibody only (result not shown).

A band of about 75 kDa detected in the plant extracts, both before and after aphid feeding (Fig. 4, lanes 2 and 3), was also detected in plant extracts when a preimmune serum was used (result not shown). Thus, it appears that the rabbit used for raising antibodies against Protein C002 had previously developed antibodies against a plant protein of 75 kDa and that these antibodies were not fully removed in passing the anti-C002 antiserum over a column of immobilized Protein C002 for antibody purification.

Effects of *c002* Transcript Knockdown on Feeding Behavior. EPG provides a powerful method to study probing behavior by an aphid during foraging and feeding (3, 13, 14). Three main phases during stylet penetration have been defined from EPG studies: the pathway phase, the xylem phase, and the phloem (or sieve element) phase (3, 14), and details within each of these phases can be discerned (3, 13–15).

We have used EPG to assess the effect on the foraging and feeding behavior of pea aphids in which transcript *c002* has been knocked down (8). Representative EPG traces are shown in Fig. 5. These (and EPGs of nine other insects in treatment and control groups) revealed dramatic differences in feeding (or attempted feeding) by the two groups. The EPGs as shown in the figure (and their continuations for 8 h) differ in several ways, most strikingly in that the *c002*-knockdown insect never entered a phloem (or sieve element) phase. In fact, of the 10 knockdown insects examined, only one exhibited a sieve element phase (and then only once and for just 30 min). The control insect, however (Fig. 5), not only entered the sieve element phase, but remained in that phase for 5 of the 8 h of observation (data not shown in the figure, where we include only the first 3 h of the trace). There are other, more subtle differences between the treatment and the control insect, for instance, a lower rate of occurrence of cell puncture signals during the probing phase.

In Table 1, we present a summary of the EPGs recorded from 10 *c002*-knockdown and 10 control-injected insects (a total of 80 h of EPGs from each group). Several foraging or feeding parameters can be seen to be affected in a statistically significant way by the knockdown. First, an aphid's ability to identify a suitable location for initiating probing is significantly reduced because it takes *c002*-knockdown aphids >6 times longer than the control insects to identify such a site and begin probing ($P =$

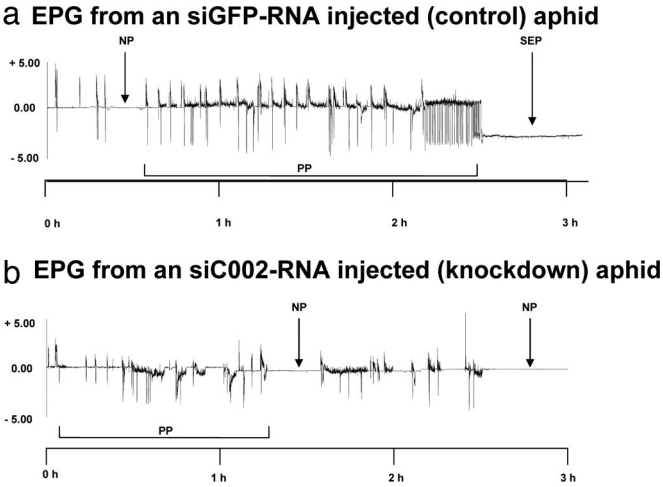

Fig. 5. Representative EPG waveforms of *c002*-knockdown insects (*a*) and control-injected insects (*b*) on fava beans. NP, nonprobing; PP, pathway phase. SEP, sieve element phase.

0.0073; Table 1). Second, once probing is initiated, the *c002*-knockdown aphids probe individual epidermal and mesophyll cells at only half the rate of control aphids ($P = 0.031$). Third and most strikingly, *c002*-knockdown aphids are far less likely to initiate sieve element phase feeding ($P < 0.0001$) than the control insects. The control aphids fed 52 times longer than the *c002*-knockdown aphids ($P < 0.0001$), which could be due either to an inability of the knockdown insects to identify and remain in a sieve element once a sieve element is probed or to simply not coming into contact with a sieve element. Finally, the *c002*-knockdown aphids spend ≈4-fold more time not engaged in probing behavior than do control insects ($P = 0.0001$).

Discussion

Here and in and a previous article (8), we have identified a protein, Protein C002, that appears to play an essential role (or roles) in the foraging and feeding of the pea aphid on fava beans, a typical host plant for this aphid species.

Protein C002 can, on the basis of our results, be considered a specialized, salivary gland protein, which does not exclude the possibility that it is synthesized in other organs in small amounts. Indeed, we have a preliminary indication that transcript *c002* occurs in gut, but at ≈100-fold lower amounts than in salivary gland (10). The organ distribution of transcript *c002* (and Protein C002) is one of the many aspects of this transcript and protein that will be under continued investigation in our laboratories.

Both Protein C002 and its transcript occur in the principal salivary glands in the pea aphid but apparently in only some of those cells (≈5 of the 21 cells within each lobe). This restriction of expression of the *c002* gene to a subset of secretory cells is intriguing. In unpublished work, we have found that the enzyme laccase is also restricted in its distribution in the principal salivary gland, but to a different subset of secretory cells than is Protein C002 (Q. D. Liang and G.R.R., unpublished observations). Thus, it appears that individual secretory cells in the principal salivary gland have different "assignments"; that is, each secretory cell will produce a particular set of proteins (many of which will be enzymes), and thus different subsets of the secretory cells could in principle produce salivas of different compositions.

Our EPG studies on *c002*-knockdown insects (and control insects) demonstrated striking effects of the knockdown. Our earlier work (8) demonstrated premature deaths of the knockdown insects feeding on fava bean leaves. The EPG results

Table 1. Analysis of the effects of *c002*-knockdown on foraging and feeding behavior in the pea aphid

Behavior examined	Control group mean (SD)	Treatment group mean (SD)	*P*, Ho: ·1 = ·2
Time to first pathway phase, min	7 (13)	45 (45)	0.0073*
Rate of PD during initial cell-probing phase, no. per min	0.50 (0.23)	0.28 (0.27)	0.031*
Total duration of pathway phase, min	260 (75)	260 (96)	0.52
Total time mouth parts not touching the plant, min	56 (40)	210 (110)	0.0001*
Time to first SEP feeding, min	130 (87)	430 (140))	<0.0001*
Total duration of SEP feeding in 8 h, min	156 (90)	3 (8.4)	<0.0001*

EPGs were recorded for 8 h per insect in each of two groups of 10: the knockdown insects, injected with siC002-RNA; and control insects injected with siGFP-RNA (see *Materials and Methods*). The statistical analysis is based on a randomization test with 10,000 iterations. PD, potential drop, caused by stylets puncturing cells; SEP, sieve element phase, observed during feeding in a sieve element.
*Significant at $\alpha = 0.05$.

reported here offer a good deal of insight into that observation. It is not much of an oversimplification to state that the knockdown aphids do not feed. They attempt to feed, but are, for all intents and purposes, unsuccessful in doing so. Thus, of 10 knockdown insects studied by EPG, only one showed a sieve element phase, and that was quite short (only 30 min). The knockdown aphids exhibit a probing phase, a typical behavior as aphids search for a sieve element (3, 13–15), but they do either not find sieve elements, do not penetrate them, or, if they do, do not maintain their penetration. In other words, the *c002*-knockdown insects essentially do not ingest phloem sap. This lack of feeding is presumably responsible for the premature death that we observed in these knockdown insects (8).

Although salivary secretions have long been recognized as vitally important in the interaction of aphids and plants (3, 7), this work describes a body of evidence that has been marshaled to demonstrate the essentiality of an individual, unambiguously identified salivary protein or enzyme and to provide direct evidence for that essentiality and for its role in feeding. The molecular mechanism by which Protein C002 acts in aphid feeding is not clear from the current results. Because Protein C002 matches no annotated protein in sequence, it is a total unknown, functionally, at the molecular level. Thus, we can exclude little at this stage. For instance, we cannot exclude that it might correspond to an enzyme activity detected by others in diluted saliva but that the enzyme occurs in the pea aphid in unrecognizable form (nonmatching amino acid sequence); we cannot exclude that it could be a calcium-binding protein. Indeed, we cannot entirely exclude the possibility that it is a structural component of the stylet sheath, although this possibility seems unlikely given the rather normal probing of epidermal and mesophyll cells exhibited by the knockdown aphids. Finally, we certainly cannot exclude a role for Protein C002 produced in another organ, especially gut, but this role would have to be in addition to that of the salivary protein, which contacts plant tissue. One possibility that intrigues us is that Protein C002 aids in identifying or maintaining contact with sieve elements and that its depletion causes a defect analogous to the inability of *Macrosiphum euphorbiae* to establish sieve element phases in transgenic potato plants having half the normal sucrose content (16). This and other possible mechanisms will be the subject of ongoing investigations in our laboratories.

Materials and Methods

Aphids, Plants, Standard Procedures, and cDNA Library Construction. The aphid colony and growth of fava beans are as described (8, 10), as are the standard methods used in this present work. The PCR-based cDNA library was made following instructions with the SMART cDNA library construction kit (Clontech), starting with RNA isolated from 250 dissected salivary glands. ESTs are posted at NCBI, accession numbers DV747494–DV752010.

Expression of Recombinant Protein in *Escherichia coli* for Antibody Preparation. A cDNA-encoding Protein C002 was amplified by PCR. We used a forward primer 5′-TAG CTG TAG CCA TGG AAG TTA GAT GCG-3′ containing an NcoI site and a reverse primer 5′-GTA TGG ACA AGC TTA TTA AAA ACG TCG-3′ containing a HindIII site. The resulting DNA encoded all 196 residues of the mature protein. The PCR product (632 bp) was ligated into a pGEM-T Easy Vector and used to transform *E. coli* strain JM109. LB/ampicillin/IPTG/X-Gal (100 μg/ml ampicillin, 0.5 mM IPTG, 80 μg/ml X-Gal) plates were used to grow transformed bacteria. White colonies were selected, and the insert was excised from the vector by digestion with NcoI and HindIII, purified by low-melting-point agarose gel electrophoresis, ligated into vector H_6pQE_{60} (17), and used to transform *E. coli* strain JM109. A single colony from the plate was used to inoculate 3 ml of 2× YT medium containing 100 μg/ml ampicillin. The culture was shaken at 300 rpm, 37°C overnight. The 3-ml overnight culture was then used to inoculate 200 ml of 2× YT medium with ampicillin and incubated at 37°C until OD_{600} was ≈0.7. Recombinant protein expression was then induced by adding IPTG to 1 mM final concentration and the culture incubated for another 5 h. Bacteria were harvested by centrifuging at 6,800 × *g*, 20 min at 4°C, and then resuspended in 4 ml of lysis buffer [8 M urea, 0.1 M NaH_2PO_4, 0.01 M Tris·Cl (pH 8.0)]. A 5-μl sample was reserved for Western blot analysis. The bacteria were incubated on ice for 30 min with Triton X-100 at a final concentration of 2% and lysozyme to a final concentration of 1 mg/ml and then sonicated on ice. The lysates were centrifuged at 12,000 × *g* for 20 min at 4°C. Supernatants and pellets were saved, and 5 μl of each sample was used for Western blot analysis.

Protein C002 expressed in 2 liters of culture medium was purified in 8 M urea by affinity chromatography with nickel-nitrilotriacetic acid (Ni-NTA) resin (Qiagen). The protein was concentrated with a YM-10 Centricon membrane (Millipore) to 400 μl, mixed with 2× SDS loading buffer, separated by electrophoresis in a 12.5% acrylamide gel (ISC Bioexpress), and stained with 0.025% Coomassie blue R-250 in water. Then, the protein band (≈600 μg) was cut out and sliced into pieces for injection into rabbit to generate antiserum (Cocalico Biologicals).

Western Blot Analysis. Polyclonal rabbit antibodies were purified on a column of immobilized recombinant Protein C002. The purified recombinant protein was linked to a matrix of cross-linked 4% beaded agarose activated to form aldehyde functional groups by using AminoLink Plus immobilization kit (Pierce). Approximately 500 aphids were placed on fresh fava bean plants and then removed after 24 h. Plant tissue (1.5 g) was homogenized in PBS in liquid nitrogen. Several freeze–thaw cycles were used, and the extract was centrifuged at 12,000 × *g* for 5 min. Approximately 2 ml of supernatant was concentrated to 200 μl by using a YM-3 Microcon centrifugal filter device (Millipore) and then filtered through a YM-50 centrifugal filter. SDS/PAGE sample buffer (6×) was added to the concentrated, filtered extract, which was then subjected to SDS/PAGE on 4–20% gradient gels (ISC Bioexpress) and transferred onto a PVDF membrane. Nonspecific binding sites were blocked with 5% instant nonfat dry milk (BestChoice), and membranes were incubated with purified polyclonal antibody (1:200 dilution) overnight followed by extensive washing for 3 h with frequent changes of PBS with 0.2% Triton X-100 (PBST). The antigen–antibody complexes were visualized with horseradish peroxidase-conjugated goat anti-rabbit IgG (Pierce) at a dilution of 1:15,000 and detected with a SuperSignal West Femto maximum sensitivity substrate kit (Pierce) on x-ray film.

Fluorescence *in situ* Hybridization. A single-stranded DNA probe was synthesized by using a PCR digoxigenin (DIG) probe synthesis kit (Roche). A 397-bp fragment of clone *c002* was amplified by PCR, and single-stranded sense or

antisense probes were synthesized by asymmetric PCR. Dissected salivary glands were fixed in 4% paraformaldehyde in PBS for 1 h at room temperature. After washing three times with PBS containing 0.2% Triton X-100 (PBST), the glands were treated with proteinase K (10 μg/ml) and fixed again with 4% paraformaldehyde for 1 h at room temperature. Prehybridization was performed, without probe, in hybridization solution (50% formamide, 5$\times$ SSC, 50 μg/ml heparin, 0.1% Tween 20, and 100 μg/ml salmon sperm DNA) at 48°C for 30 min. The fixed glands were exposed to DIG-labeled single-strand sense or antisense DNA probes (200 ng) in hybridization solution at 48°C for 20–30 h and washed successively in hybridization solution and in PBST. The glands were then blocked with 1% BSA by incubating at room temperature for 30 min, washed, and incubated with anti-digoxigenin horseradish peroxidase overnight at 4°C. After another wash in PBST, the samples were exposed to the Tyramide signal amplification kit (Molecular Probes).

Nuclei were counterstained with 1.5 μM propidium iodide (Molecular Probes) for 30 min at room temperature. Finally, the glands were mounted in glycerol and examined under a fluorescence microscope (Nikon Eclipse E800) with a triple bandpass filter for FITC (green color) and Cy3 (red color). Photographs were taken with a digital camera attached to the compound microscope, and pictures were edited with Adobe Photoshop 7.0.

Immunohistochemistry. Pea aphid salivary glands were dissected in PBS, washed three times in PBST, and fixed in Bouin's fixative (71% saturated picric acid, 24% formaldehyde, 5% glacial acetic acid) for 10 min at room temperature in a humidified chamber. The glands were washed extensively with PBST and incubated with primary antibody (raised in rabbit against recombinant C002) at 1:100 dilution overnight at 4°C. The glands were then washed three times at 15-min intervals with PBST and blocked with 5% normal goat serum in PBST for 1 h, washed three times at 15-min intervals with PBST, and incubated with secondary antibody Cy3-conjugated goat anti-rabbit (Jackson ImmunoResearch Laboratories) at 1:500 dilution overnight at 4°C. The glands were then washed extensively with PBST at 15-min intervals. Nuclei were stained by using TO-PRO-3 (Molecular Probes, Invitrogen) at 5 μM for 30 min in dark at room temperature. Glands were washed extensively with PBST and mounted on mounting media (Gel/Mount; Biomeda Corporation) on a glass slide. Photographs were taken with a Nikon Zeiss LSM 5 Pascal (laser scanning confocal microscope).

Analysis of Aphid Feeding Behavior. EPG measurements were carried out in an electrically grounded Faraday cage to shield the setup from external electrical noise. All experiments were carried out at room temperature (22°C– 24°C). A gold wire, 2 cm long and 10 μm in diameter, was glued to the dorsum of the aphid by using conductive silver paint (Colloidal Silver; Ted Pella, Inc.). The wiring was done by immobilizing the aphid with a vacuum-operated plate. The gold wire (insect electrode), which allowed some free movement by the aphid on the plant's surface, was connected to the EPG probe. A stiff copper wire, 10 cm long and 0.2 cm in diameter, from the EPG monitor, was inserted into soil of the pot in which the plant was rooted (plant electrode). The two electrodes were connected to an eight-channel GIGA-8 direct current amplifier (Wageningen Agricultural University, Wageningen, The Netherlands), which has 10^9 Ω input resistance and an adjustable plant voltage. When the aphid stylets come into contact with the electrified plant, the circuit is closed, and current flows through the insect and into the monitor, thus generating an interpretable signal. The feeding behavior of individual aphids on fava bean plants was monitored for 8 h with the help of a four-channel amplifier (two channels for siGFP-RNA-injected aphids and two for siC002-RNA-injected aphids). Ten replications were completed for each of two groups. The target group was *c002*-knockdown insects, injected as described in ref. 8 and maintained for 3 days on an artificial diet. These conditions produce $\approx$60% knockdown of transcript *c002* (figure 3 in ref. 8). The control group consisted of insects injected with siGFP-RNA (8) and otherwise treated the same as the target group. Waveform recordings were analyzed with the EPG analysis software PROBE 3.0 installed on a PC.

The parameters measured in this work included: (*i*) time to the first pathway phase (a measure of how quickly an aphid can identify a suitable location for probing individual cells); (*ii*) the rate of occurrence of potential drops during the initial pathway phase (a measure of how quickly an aphid probes individual plant cells with its stylet); (*iii*) the total duration of the pathway phase (a measure of time spent searching for a nutrient-rich sieve element); (*iv*) time to first sieve element phase feeding (a measure of how quickly an aphid can find a nutrient-rich cell for feeding); and (*v*) total duration of sieve element phase feeding (a measure of time spent feeding on a nutrient-rich source). If the sieve element phase is not reached during the entire experiment time, the time to first sieve element phase was considered to equal the total observation time (15), i.e., 8 h for this work. We tested for significant differences between our two treatments by using a randomization approach with 10,000 iterations. Specifically, we tested the null hypothesis that Ho: $\mu_{\text{siGFP-RNA}} = \mu_{\text{siC002-RNA}}$ by randomizing the data and determining the probability of getting the observed $\mu_{\text{siC002-RNA}}$. We used the SAS platform (18) and $\alpha = 0.05$.

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